

Targeted molecular analysis in adrenocortical carcinomas

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Clinical Research Article

Targeted molecular analysis in adrenocortical carcinomas: a strategy towards improved personalized prognostication.

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Short title: Precision medicine in ACC

Key words: adrenal cancer, endocrine-related cancer, molecular oncology, prognosis, drug targets, personalized medicine.

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33

34 **Precis:** We established a clinically applicable method to simultaneously improve disease risk
35 assessment and identify drug targets paving the way to a precision medicine approach in
36 adrenocortical carcinoma.

Abstract

Context: Adrenocortical carcinoma (ACC) has a heterogeneous prognosis and current medical therapies have limited efficacy in its advanced stages. Genome-wide multi-omics-studies identified molecular patterns associated with clinical outcome.

Objective: Here, we aimed at identifying a molecular signature useful for both personalized prognostic stratification and druggable targets, using methods applicable in clinical routine.

Design: 117 tumor samples from 107 ACC patients were analyzed. Targeted next-generation sequencing of 160 genes and pyrosequencing of 4 genes were applied to formalin-fixed paraffin-embedded (FFPE) specimens to detect point mutations, copy number alterations and promoter region methylation. Molecular results were combined with clinical/histopathological parameters (tumor stage, age, symptoms, resection status, and Ki67) to predict progression-free survival (PFS).

Results: In addition to known driver mutations, we detected recurrent alterations in genes not previously associated with ACC (e.g. *NOTCH1*, *CIC*, *KDM6A*, *BRCA1*, *BRCA2*). Best prediction of PFS was obtained integrating molecular results (>1 somatic mutation, alterations in Wnt/ β -catenin and p53 pathways, high methylation pattern) and clinical/histopathological parameters into a combined score ($P<0.0001$, chi-square 68.6). Accuracy of prediction for early disease progress was 83.3% (area under the ROC curve: 0.872, 0.80-0.94). Furthermore, 17 potentially targetable alterations were found in 64 patients (e.g. in *CDK4*, *NOTCH1*, *NF1*, *MDM2*, *EGFR* and in DNA repair system).

Conclusions: This study demonstrates that molecular profiling of FFPE tumor samples improves prognostication of ACC beyond clinical/histopathological parameters and identifies new potential drug targets. These findings pave the way to precision medicine in this rare disease.

Introduction

Adrenocortical carcinoma (ACC) is a rare tumor with a generally poor, but heterogeneous prognosis (5-year survival rate ranging from 13% to 80% [1 2]). Tumor stage according to the ENSAT (European Network for the Study of Adrenocortical Tumors) classification, which has now been used also by the American Joint Committee on Cancer (AJCC) TNM (tumor, nodes, metastasized) classification system (**Supplemental Table 1** [3], <https://www.cancer.org/cancer/adrenal-cancer/detection-diagnosis-staging/staging.html>), is one of the most relevant prognostic factors [1]. However, about 10% of patients with metastatic disease at diagnosis are still alive after 10 years and more than 20% of patients with tumor stage I-III die within the first three years [1]. Resection (R) status of the primary tumor [4 5] and Ki67 index [6] represent additional prognostic factors. A recent study also proposed a combination of clinical/histopathological parameters (i.e. tumor grade, R status, age, symptoms=GRAS score) to improve prognostication in patients with advanced ACC [7].

Genome-wide studies have identified molecular patterns associated with clinical outcome [8 9]. Among these, a specific gene expression pattern (i.e. high *BUB1B-PINK1* levels) [8 10], specific copy number (CN) alteration [9] and CpG islands methylation patterns [8 11] have been associated with a poor prognosis. However, these studies have been performed on fresh-frozen tumor samples that are difficult to collect in routine clinical settings. Moreover, cost-intensive genome-wide technologies and complex bioinformatics workup were required, which precludes the adoption of the proposed prognostic biomarkers in clinical practice.

At present, few effective pharmacological therapies are available for ACC[12]. Mitotane (Lysodren, Bristol-Myers Squibb, Princeton, New Jersey, USA) is the only approved drug, but an objective response is observed in only ~20% of cases and treatment is limited by severe adverse reactions [13 14]. Even combined therapies of mitotane and cytotoxic chemotherapies, like etoposide-doxorubicin-cisplatin (EDP) [15], streptozotocin [15] and gemcitabine plus capecitabine [16 17], exhibit response rates <25%. Although some studies provided some promising insights into potential pharmacological targets [18-20], effective targeted therapies have not been identified yet [2 21].

The main aim of the present study was to identify a molecular tumor signature for a prognostic classification of ACC patients that may be easily transferred into clinical practice. To this end, we

used 117 standard formalin-fixed paraffin-embedded (FFPE) tumor tissue specimens to investigate the prognostic power of both previously proposed or new molecular markers and potential drug targets, which we evaluated by targeted next-generation sequencing (NGS).

Patients and Methods

Study protocol: This is a single-center retrospective study designed and conducted in accordance with the Declaration of Helsinki. We followed the recommendations for tumor prognostic markers studies reported in REMARK [22]. Moreover, we searched for known drug targets using the OncoKB website (<http://oncokb.org/#/> [23]). The study protocol was approved by local ethics committee (#88/11) and written informed consent was obtained from all subjects prior to study enrollment.

Patient cohort and clinical data: 107 patients were selected for the study. Inclusion criteria were histologically confirmed diagnosis of ACC and availability of FFPE tumor specimens collected between 2002 and 2016 and corresponding blood samples. Initial clinical/histopathological parameters and follow up data were collected through the ENSAT registry (<https://registry.ensat.org/>) (**Table 1**).

A modified version of the GRAS classification [7] (mGRAS score) was used to merge prognostically relevant clinical/histopathological data: tumor stage (ENSAT 1-2=0 points, 3=1 point, 4=2 points), grading (Ki67 proliferation index 0-9=0 points, 10-19=1 point, ≥ 20 =2 points), resection status (R0=0 points, RX=1 point, R1=2 points, R2=3 points), age (< 50 years=0 points, ≥ 50 years=1 point) and symptoms due to steroid autonomous secretion or tumor mass (no=0 points, yes=1 point).

Progression-free survival (PFS) was the major outcome being defined as the time from tumor resection (primary surgery) to first radiological evidence of disease relapse or disease-related death.

Material collection and DNA isolation: The final series included 117 FFPE samples (89 primary tumors, 10 local recurrences and 18 distant metastases). In 10 cases, tumor tissues were available from consecutive surgeries of the same patients (7 with primary+metastasis, one with primary+local recurrence, one with local recurrence+metastasis, one with two metastases). For survival analyses only the chronologically first sample of a patient was used (either primary tumor or local

recurrence/metastasis). The tumor cell content in each FFPE slide was assessed by hematoxylin-eosin staining and reached a high fraction (median 90%, range 60-95%). DNA was isolated from tumors with the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) and from peripheral blood with the NucleoSpin Blood L Kit (Macherey-Nagel, Bethlehem, PA, USA) according to the manufacturer's instructions. Qualitative and quantitative evaluation of DNA fragmentation was assessed by the GeneRead DNA QuantiMIZE Assay Kit (384) (Qiagen). qPCRs were performed with a SYBRGreen mix according to the manufacturer's protocol and measured with a ViiA7 Real-Time PCR (RT-PCR) System (Thermo Fisher Scientific, Manassas, VA, USA). Data were analyzed with QuantStudio™ RT-PCR Software (Applied Biosystems, Foster City, CA, USA). The quality of all DNA samples was calculated with GeneRead DNA QuantiMIZE_384_DataAnalysis (Qiagen) in comparison to a control DNA included in the kit. Only DNA with a QC Score (indicator of sample damage/fragmentation) ≤ 0.04 was sequenced.

Targeted DNA sequencing: Tumor and leukocyte DNAs were enriched with the GeneRead DNaseq Human Comprehensive Cancer Panel V2 and GeneRead DNaseq Panel PCR Kit V2 (both Qiagen), according to the manufacturer's protocol. This panel includes coding regions of 160 genes (7951 amplicons and 744835 bases of target regions), many of them known or suspected to be involved in adrenocortical tumorigenesis or known drug targets. NGS was performed on a NextSeq500 with NextSeq Mid Output Reagent Kit V2 and 150bp paired end reads (Illumina Inc, San Diego, CA, USA). Raw data were aligned and analyzed with GensearchNGS (Phenosystems S.A., Belgium). For detection of somatic point mutations and small insertions and deletions (small Indels) in tumor samples the called variants were filtered as followed: coverage >100 ; exon distance <21 ; frequency of appearance >0.1 ; Minor Allele Frequency (MAF) <0.02 ; variant balance >0 . Variants found also in the matched blood samples were excluded. Intronic and synonymous variants have been considered for calculating Tumor Mutational Burden (TMB) but not for further analysis, except of those with a predicted influence on splice sites.

Impact on splicing for intronic and synonymous variants was analyzed with Alamut software (Interactive Biosoftware, Rouen, France) using five prediction algorithms: SpliceSiteFinder-like,

MaxEntScan [24], NNSPLICE [25], GeneSplicer [26] and Human Splicing Finder [27]. All other variants were evaluated for predicted pathogenicity by Polymorphism Phenotyping v2 algorithm tool (PolyPhen-2) (<http://genetics.bwh.harvard.edu/pph2>) [28], SIFT (Sorting Tolerant From Intolerant) algorithm (<http://sift.jcvi.org/index.html>) and MutationTaster (<http://www.mutationtaster.org/>) [29]. The Catalogue Of Somatic Mutations In Cancer (COSMIC) was used as a reference of cancer-related somatic variants (<http://cancer.sanger.ac.uk/cosmic>). InterVar was used as an additional tool for the interpretation of variants [30].

ZNRF3, which was previously reported to be involved in the pathogenesis of ACC [8 9 31], was evaluated separately by direct Sanger sequencing. PCR primers for the coding region of *ZNRF3*, except exon 1, were designed with Primer3 (version 4.0.0) software [32] (**Supplemental Table 2 [3]**). Sequencing data were generated with an ABI 3730 or an ABI 3130xl capillary sequencer under standard conditions and analyzed with Gensearch (Phenosystems S.A.).

TMB was calculated by summing up all detected somatic variants and dividing this number by the size of the target region. Values are specified in variations per megabase (Mb).

To analyze CNAs we used a combination of two different approaches: the CNV analysis tool from GensearchNGS and an in-house pipeline. Single CN gains or losses have been identified by comparing CN of matched tumor and blood samples. With GensearchNGS a fold change (FC) of 1 was considered as “normal”. Hence genes with all amplicons having a FC between 1.25 and 1.75 were considered as “heterozygous duplicated” and as “homozygous duplicated” with a FC >1.75. Genes were considered as deleted when all amplicons showed a FC <0.75. For the second approach an initial quality assessment was performed using FastQC, v0.11.3 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Adapters and low quality reads were trimmed using TrimGalore, v0.4.0 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) powered by Cutadapt, v1.8 (<https://cutadapt.readthedocs.io/en/stable/>). The reads were aligned against the UCSC hg19 human reference genome with BWA mem, v0.7.12 (<http://bio-bwa.sourceforge.net/>) using default parameters [33]. Sorted BAM-files were created using Picard v1.125 (<http://broadinstitute.github.io/picard>) and indexed using SAMtools v1.3 (samtools.sourceforge.net/) [34]. Local realignment around Indels was executed with GATK, v3.5

(<https://software.broadinstitute.org/gatk/>) [35]. For CNV calling the number of reads of each amplicon was determined using the multiBamCov-Tool in the BEDTools suite, v2.26.0 (bedtools.readthedocs.io/) [36]. Only markers covered with an average of at least 200 reads in control samples in the respective panel were considered. For normalization, the reads for each amplicon were divided by the total number of reads for each sample. Log2 FC was calculated for each amplicon passing QC using the corresponding amplicon in the matched control. A gene was considered amplified or deleted if at least 80% of all markers in a tumor covering the gene were amplified or deleted at least 1.5-fold. In both approaches, CNAs were only investigated for genes covered by at least 6 probes. Only CN alterations detected with both approaches were considered.

Targeted DNA methylation analysis: Bisulfite pyrosequencing was used for quantitative methylation analysis of four tumor suppressor genes *PAX5*, *PAX6*, *PYCARD* and *GSTP1* that have been demonstrated to play a significant prognostic role in ACC [11]. 500 ng of DNA from tumor and matched blood samples was used to perform bisulfite conversion and cleanup of converted DNA with the EpiTect®Fast 96 DNA Bisulfite Kit (Qiagen) according to the manufacturer's protocol. Target regions of the assays were selected to include the regions accessible with the MLPA ME002 tumor suppressor-2 probe mix (MRC-Holland, Amsterdam, The Netherlands) (**Supplemental Figure 1 [3]**). PCR and sequencing primers were designed with PyroMark Assay Design 2.0 software (Qiagen) (**Supplemental Table 3 [3]**). Bisulfite-treated DNA was amplified in 25µl reactions containing 2.5µl 10xPCR buffer with 20mM MgCl₂, 0.5µl 10mM dNTP mix, 1.0µl (10pmol) of each forward and reverse Primer, 0.2µl FastStart Taq DNA Polymerase (5U/µl), 18.8µl PCR-grade H₂O and 1µl bisulfite-converted DNA. PCR was carried out with an initial denaturation step at 95°C for 5 min, followed by 45 cycles 95°C for 30 seconds, primer-specific annealing temperature (58°C for *PAX5* and *PYCARD*, 59°C for *PAX6*, and 60°C for *GSTP1*) for 30 seconds and elongation at 72°C for 30 seconds and a final extension step at 72°C for 7 min. Bisulfite pyrosequencing was performed on a PyroMark Q96 MD Pyrosequencing System with the PyroMark Gold Q96 CDT Reagents Kit (Qiagen). Pyro Q-CpG software (Biotage, Uppsala, Sweden) was used for data analysis.

FISH analysis: To validate *CDK4* CN gains, we investigated 6 representative 2µm-thick FFPE slides by FISH analysis (two samples with “homozygous” *CDK4* amplification, two with “heterozygous” *CDK4* amplification and two with normal *CDK4* allele status at NGS). *CDK4* gene amplification was visualized through hybridization of a Zytolight SPEC *CDK4/CEN12* Dual Color Probe (ZytoVision GmbH, Germany) (D12Z3) according to the manufacturer’s recommendation. At least 200 non-overlapping nuclei per sample were evaluated by fluorescence microscopy (Zeiss Axioskop, Jena, Germany) using the appropriate filter sets. Only nuclei with a distinct nuclear border showing clear hybridization signals were evaluated. *CDK4* gene was considered heterozygous amplified when the FISH signal ratio of *CDK4/CEN12* was between 1.0 and 2.0 or homozygous amplified when the ratio was ≥ 2.0 . Ratios may nevertheless differ when gains affect whole chromosome 12.

Targeted gene expression analysis: The mRNA expression of *BUB1B* and *PINK1* was evaluated by quantitative real-time RT-PCR only in samples with high quality RNA and cDNA (n=38). All baseline clinical/histopathological characteristics as well as follow up data of this subgroup of patients did not differ from those of the entire series. RNA was isolated from tumors by miRNeasy FFPE (Qiagen). High RNA quality was tested using an Agilent 2100 Bioanalyzer (RIN >7.5). RNA was reverse transcribed by the Quantitec Reverse Transcription Kit (Qiagen). A quantitative RT-PCR for *β-actin* and *GAPDH* was performed and only samples with cycle threshold of <35 were included from further analysis (n=38). The expression of *BUB1B* and *PINK1* was evaluated by qRT-PCR using Taqman® *BUB1B* (Hs01084828_m1) and *PINK1* (Hs00260868_m1) probes with expressed *β-actin* (Hs9999903_m1) as reference (Applied Biosystems, Darmstadt, Germany). Each PCR reaction was done with 40 ng cDNA and each analysis was performed in duplicate. Transcript levels were determined using the TaqMan Gene Expression Master Mix (Applied Biosystems), the CFX96 real-time thermocycler (Biorad, Hercules, CA, USA), and the Bio-Rad CFX Manager 2.0 software. Cycling conditions were 95 °C for three min followed by 40 cycles of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec. The ΔCT method was applied for normalization of gene expression levels to those of *β-actin*. The $\Delta CT(BUB1B) - \Delta CT(PINK1)$ expression was then calculated [10]. Statistical analysis with different cut-off values was performed. The best cut-off value for a high *BUB1B-PINK1*

differential expression was 6.3, which was already previously suggested by Bertherat and colleagues [10].

Statistical analysis: A Fisher's exact or Chi-square test was used to investigate dichotomic variables, while a two-sided *t* test (or Mann-Whitney non-parametric test) was used to compare two groups of continuous variables as appropriate. A non-parametric Kruskal-Wallis test, followed by Bonferroni *post-hoc* test, was used for comparison among several groups for non-normal distributed variables. Correlations and 95% confidence intervals (95%CI) between different parameters were evaluated by linear regression analysis. Survival curves were obtained by Kaplan-Meier estimates and the differences between two or more curves were assessed by the log-rank (Mantel-Cox) test. Multivariate regression analysis was performed by Cox proportional hazard regression model to identify those factors that might independently influence survival.

To assess and compare the prognostic accurateness and performance of different markers or scores we used two approaches: 1) We utilized the chi-square (log-rank) values (deviance chi-square test) to determine the goodness-of-fit statistic of the regression model, representing a surrogate of a Likelihood Ratio Test. 2) We calculated sensitivity, specificity and accuracy of different models categorizing patients with or without disease recurrence/progress within 24 months from primary surgery as affected/non affected. Finally, we considered the area under the receiving operating characteristics (ROC) curve and 95% confidence interval for predicted probability of disease progress within 24 months from primary surgery.

Statistical analyses were made using GraphPad Prism (version 6.0, La Jolla, CA, USA) and SPSS Software (version 23, SPSS Inc., Chicago, IL, USA). P values <0.05 were considered as statistically significant.

Results

Targeted molecular analysis of ACC: overview

The clinical and histopathological characteristics of the 107 patients selected for the study are shown in the **Table 1** (see also Patients and Methods).

By performing targeted NGS in 117 ACC samples, we found a median **TMB** of 1.3/Mb (range: 0-22,8/Mb). Altogether, we found 237 somatic genetic variants (SNVs and small Indels). The complete list of alterations and their characteristics is shown in **Supplemental Data** [3]. Considering the 10 cases with FFPE samples from consecutive surgical interventions, most variants in driver genes were conserved in samples obtained from same patient (**Supplemental Figure 2** [3]). Thus, we considered only the first available sample from each of the 107 ACC patients. Among them, 30 presented no mutations, 25 one mutation and 52 at least two mutations (median per sample: 1, range: 0-14, ≥ 5 mutations in 13 cases). Overall, 215 protein-altering somatic variations were found, affecting 69/161 evaluated genes. Among the affected genes, 40 were mutated in at least two samples and 17 in at least three samples (frequency $\geq 2.8\%$) (**Supplemental Table 4** [3]). The frequency of recurrent mutations previously described in ACC [8 9 31] and in our series is shown in **Figure 1A**. The most frequently mutated genes were: *TP53* (22%), *CTNNB1* (17%), *NF1* (11%), *APC* (8.4%), *ZNRF3* (8.4%), *MEN1* (7.4%), *GNAS* (6.5%) and *ATRX* (6.5%). We also discovered novel recurrent mutations not clearly associated with ACC yet, such as in *NOTCH1*, *CIC*, *KDM6A*, *BRCA1* and *BRCA2* (all $\geq 2.8\%$) (**Figure 1A and Supplemental Table 4** [3]).

We then evaluated somatic CNVs in the same series. Most frequent CN gains were observed in *CDK4* (43%), *NOTCH1* (19%), *TERT* (12%), *FGFR3* (12%) and *MDM2* genes (7.4%) and CN losses at *RBI* (5.6%), as expected (**Figure 1B**). The presence of amplifications at *CDK4* locus was confirmed by FISH analysis (see **Figure 2**). We also found CN alterations that were not previously reported in ACC, such as gains in *STK11* (31%), *GNAI1* (17%) and losses in *TNFRSF14* (30%), *SMARCB1* (22%), *FLCN* (20%) and *CHEK2* (13%) (**Figure 1B**).

Using our targeted sequencing approach, we identified three different CN patterns, consistent with a previous report [9]. Accordingly, we defined them as “chromosomal” when at least three large chromosomal regions were affected by amplifications or deletions, “quiet” when less than three

regions were altered and “noisy” when several small regions were affected (modified from [9]). An example of each CN pattern is reported in **Supplemental Figure 3** [3]). 38 ACC samples were recognized to present a “chromosomal” pattern, 44 a “quiet” pattern and 25 a “noisy” pattern.

The two most frequently affected pathways were p53/Rb signaling (59.8%; including alterations in *CDKN2A*, *CDK4*, *MDM2*, *RB1* and *TP53*) and Wnt/ β -catenin pathway (33.6%, including alterations in *APC*, *CTNNB1*, *MED12*, *MEN1* and *ZNRF3*). In 22 of 107 samples (20.6%) both pathways were involved. 3 of these patients (2.8%) had variations in *CTNNB1* and *TP53*. Another frequently altered pathway was the chromatin remodeling pathway (29.9%) (Fig 1C). In a lower percentage of cases, genetic variations in genes of the DNA repair (7.4%) or the mismatch repair (MMR) systems (4.5%) were observed (**Figure 1C**).

The methylation pattern of promoter regions of four preselected genes was also evaluated. The median percentage of methylated promoter regions in the tumor material was 11% at *PAX5* (range: 1-98), 22% at *PAX6* (range: 2-97), 17% at *PYCARD* (range: 1-94) and 3% at *GSTP1* (range: 1-74). Considering all genes, the median value of mean methylation was 21% (range: 2-77). 33 tumors presented a promoter methylation status “high” (31% of cases).

A high *BUB1B-PINK1* differential expression is a known negative prognostic marker in ACC [10]. Thus, we evaluated *BUB1B* and *PINK1* mRNA expression levels in a subgroup of 38 FFPE tumor specimens with good RNA quality (32.5%). The analysis of this series revealed a high *BUB1B-PINK1* differential expression in 16 cases (42%, **Supplemental Figure 4**).

Prognostic stratification

To evaluate the benefit of applying a molecular classification to prognosticate clinical outcome, we first investigated prognostic effectiveness of ENSAT tumor staging classification in our series. As expected, the median PFS was shorter for patients with metastatic disease (ENSAT 4, n=23) than for those with intermediate (ENSAT 3, n=28) or early tumor stages (ENSAT 1 to 2, n=58) ($P<0.0001$, chi-square=35.6, **Figure 3A**). However, using the mGRAS score (see Methods), we obtained an improved prognostic stratification by recognizing 4 subgroups with different clinical outcome, from

favorable prognosis (median PFS=54 months) to poor prognosis (median PFS=3 months) ($P<0.0001$, chi-square=49.0, **Figure 3B**).

Considering the results of the targeted molecular analysis, five events predicted a shorter PFS in univariate analysis: 1. Presence of more than one mutation ($P=0.0015$, HR=2.12, 95%CI=1.3-3.4); 2. Noisy CNA pattern ($P=0.0038$, HR=2.46, 95%CI=1.3-4.5); 3. Presence of alterations in Wnt/ β -catenin signaling alone or together with p53/Rb ($P<0.0001$); 4. Promoter methylation status “high” ($P=0.0002$, HR=2.9, 95%CI=1.7-5.0); 5. High *BUB1B-PINK1* differential expression (n=38, $P=0.0037$, HR=2.56, 95%CI=1.16-5.67). To investigate the applicability of a molecular prognostic classification in a clinical setting, we developed a simplified score excluding parameters that cannot be reliably and easily analyzed by targeted analysis in FFPE samples (i.e. CNA pattern and mRNA expression). At multivariate analysis including clinical/histopathological parameters, presence of alterations at Wnt/ β -catenin alone or with p53/Rb signaling and promoter methylation status “high” remained significant ($P=0.026$, HR=1.39, 95%CI=1.04-1.87, and $P=0.003$, HR=2.03, 95%CI=1.27-3.25, respectively). We then combined genetic items in a molecular score as follows: number of somatic mutations (0-1=0 points, >1=1 point), alterations in the Wnt/ β -catenin and p53/Rb pathways (none=0 points, only Wnt/ β -catenin=1 point, Wnt/ β -catenin+ p53/Rb=2 points) and promoter regions methylation pattern ($\leq 25\%$ =0 points, >25%=1 point) (overall points 0 to 4). This allowed us to separate four groups with PFS as endpoint: score 0 (n=35, median PFS=36 months), score 1 (n=30, median PFS=9 months), score 2 (n=22, median PFS=6 months) and score 3-4 (n=20, median PFS=4 months) ($P<0.0001$, chi-square=34.4; for definition see **Figure 3C**).

By merging mGRAS and molecular score into a combined (COMBI) score, we obtained a further improvement in the progression risk stratification. In particular, we better distinguished a group of patients with a really favorable prognosis (median PFS=54 months) and further three groups with good (median PFS=13 months), intermediate (median PFS=6 months) and poor prognosis (median PFS=3 months) ($P<0.0001$, chi-square=68.6; for definition see **Figure 3D**). When we tested the superiority of COMBI in respect to mGRAS score by discriminating patients with the best clinical outcome (at least 24 months free of disease progression), COMBI score showed a better prognostic performance, proven by superior specificity (58.6 vs 31.0%) and accuracy (83.3% vs 74.5%).

Moreover, the area under the ROC curve was higher for COMBI than for mGRAS score (0.872, 95%CI=0.800-0.943, vs 0.780, 95%CI=0.689-0.871) (**Supplemental Figure 5A-B** [3]).

A heatmap sorted for prognosis including mGRAS score, molecular parameters and COMBI score is shown in **Figure 4**.

We then decided to compare the prognostic power of mGRAS and COMBI score evaluating the disease-free survival (DFS) in those 74 ACC patients that were successfully operated (R0). In this subgroup, only COMBI score was able to identify a category of patients with an extremely longer DFS: median DFS for COMBI 0-2 (n=23)=243 months, COMBI 3-4 (n=30)=13 months, COMBI 5-7 (n=18)=5.5 months, COMBI 8-13 (n=3)=3 months ($P < 0.0001$, Chi-Square 50.98, see **Supplemental Figure 6**).

Prediction of response to therapy

In patients treated with adjuvant mitotane (n=39), a low COMBI score (0-2) was slightly more powerful to predict a longer disease-free survival than a low mGRAS (0-1) ($P=0.0001$, chi-square=21.5, vs $P=0.0058$, chi-square=12.5). However, similar results were obtained considering patients with superimposable disease stages that did not receive adjuvant mitotane (n=49) (COMBI score: $P=0.0001$, chi-square=27.5, mGRAS: $P=0.0008$, chi-square=16.8), thus suggesting no specific relationship between molecular alterations and response to mitotane.

In patients with advanced ACC, none of the single molecular events showed a significant predictive role for response to mitotane monotherapy (n=34), EDP-M (n=52), gemcitabine plus capecitabine (n=36) and/or streptozotocin (n=44). These analyses were performed by considering both objective response to the investigated drugs and time to progression during treatment.

Actionable molecular alterations

Having chosen a NGS panel that includes several known pharmacologically targetable genetic alterations allowed us to directly look for their presence in ACC. According to the level of evidence (OncoKB website), we found at least one alteration in a drug targetable gene in 64 of our 107 patients. The list and specifics of 17 actionable genetic alterations are reported in **Table 2**. Most interesting

ones are CN gains at gene *CDK4* (43% of cases) that are accessible by different CDK4/6 inhibitors already approved for other types of solid tumors. Moreover, recurrent alterations at *NOTCH1*, targeted by gamma secretase inhibitors, *NF1*, targeted by MEK inhibitors, or at *MDM2*, targeted by MDM2 inhibitors, were recognized. Mutations in other known druggable genes, such as those coding for receptor tyrosine kinases (*EGFR*, *KIT*, and *RET*), members of the DNA repair system (*ATM*, *BRCA1*, and *BRCA2*), *PTCH1* and *TSC1/TSC2* were detected in a small percentage of samples (<3%). In two ACC samples we identified the well-known Val600Gly activating mutation in the gene *BRAF*, which is found in approximately 50% of papillary thyroid carcinomas and is directly actioned by BRAF and/or MEK inhibitors. Finally, mutations and/or CN losses were also observed in MMR genes *MLH1*, *MSH2*, and *MSH6*, which are associated with response to immune checkpoint inhibitors such as PD1/PDL1 inhibitors.

Discussion

The present study represents the largest study combining targeted NGS and methylation analysis on ACC samples (n=117) using FFPE tissue specimens that are easily obtainable during routine histopathological workup. Our results clearly demonstrate that these analyses are feasible on FFPE material. Furthermore, we propose a new combined histological, clinical, and molecular score that improves the prognostic stratification in this rare disease (COMBI score). Finally, we identify actionable molecular events in 60% of patients.

Interestingly, we could evaluate the genetic profile of consecutive tumors from 10 patients. In these cases, we found a good concordance between primary and recurrent tumors in terms of both TMB and mutated genes, similarly to what is described for other cancer types [37]. Thus, we considered only the first available tumor sample for each single patient (n=107). Overall, we confirmed the presence of frequent ACC-associated alterations (**Figure 1A**). Notably, we also detected in a smaller percentage of cases alterations previously not clearly associated with ACC (>2.5%, i.e. mutations at *NOTCH1*, *CIC*, and *BRCA1/2*, amplifications in *STK11*, *GNA11* and deletions in *TNFRSF14*, *SMARCB1*). In terms of signaling pathways, the most frequently involved were p53/Rb and Wnt/ β -catenin, as expected. In 22 samples (20.6%) we observed alterations in both signaling pathways representing an important negative prognostic marker - a rate that was already reported in literature [8 9]. In the group with worst prognosis also 3 patients (2.8%) with alterations in *CTNNB1* and *TP53* were observed. While Ragazzon et al. found alterations in *CTNNB1* and *TP53* mutually exclusive, a small number of patients in the cohort of Assié et al. and Zheng et al. had variants in both genes, thus also supporting our data. We also found alterations in genes involved in chromatin remodeling, as expected [8 9 31 38]. More surprisingly, we also observed recurrent genetic alterations affecting members of the MMR (i.e. *MLH1*, *MSH2*, *MSH6*) or homologous recombination DNA repair system (i.e. *ATM*, *BRCA1*, *BRCA2*).

Concerning the prognostic role of molecular markers, we could confirm in our FFPE series the impact of already proposed molecular markers already proposed in studies on fresh-frozen material [8 9 31]. However, the investigation of the CN pattern was not easily achievable starting from targeted analysis in FFPE material. Similarly, the isolation of high quality RNA from FFPE tissue was

successful in only 32.5% of samples, allowing investigation of mRNA expression in only a subset of patients. Therefore, we excluded these markers from further analysis. A simplified molecular prognostic score was then devised which includes mutational load, alterations in p53/Rb and Wnt/ β -catenin pathway and promoter methylation status “high”. However, importantly, only by merging molecular alterations with clinical/histopathological parameters included in mGRAS into a COMBI score, we obtained the best discrimination among ACC patients with different prognostication. COMBI score was particularly supportive to identify patients with an extremely favorable clinical outcome, showing the best predictive accuracy for discriminating patients without disease recurrence/progress within the first 24 months after primary surgery **when compared to mGRAS score.** **The superiority of COMBI score was even more evident when considering the capability to predict DFS in patients successfully operated.** These findings might play a key role in clinical practice, helping to better select patients that do not need aggressive treatment, thus sparing unnecessary side effects to patients and costs for the community.

A targeted approach to molecular analysis has been recently proposed by Assie and colleagues [39], who validated targeted NGS for calling simultaneously mutations, chromosome alterations and DNA methylation status. Such analysis might have clinical benefits, but still needs to be validated in FFPE material. **Considering other cancer types, genetic analysis by targeted NGS and methylation analysis by pyrosequencing have been performed in FFPE tumour specimens obtaining good results [40-42]. Nevertheless, this kind of approach had not been tested in ACC samples until now.** In general, it is now the task to prove that proposed molecular-driven scores are clinically helpful to guide clinicians in patient care. To this end, only a multicenter, prospective and randomized trial will provide reliable answers, but the international ACC community seems to be well connected to perform such effort.

Furthermore, we intended to investigate the potential predictive role of molecular alterations for response to systemic chemotherapies. However, none of the evaluated alterations were associated with the response to any standard pharmacological therapy in ACC. This might have different explanations, including the heterogeneity of treatments usually used in this kind of patients and the complexity of the molecular background of ACC.

Finally, we intended to identify potentially druggable molecular events. A similar approach has been used in few previous studies in small series of patients (up to 40) demonstrating the presence of potentially actionable genomic alterations in a subset of ACC [19 20]. In our study, we concentrated on molecular events targeted by drugs already available for solid tumors (OncoKB). Based on our analysis, the most promising candidate is the gene *CDK4*. Specifically, CN gains at the *CDK4* locus are already reported in the literature on ACC [8 19 20], but we observed them in an even higher percentage of cases (>40%). These alterations were confirmed with FISH analysis. Our findings may be clinically relevant because selective CDK4/6 inhibitors palbociclib and ribociclib have been approved by FDA for treatment of breast cancer [43]. Phase I-III studies are now ongoing with other CDK4/6 inhibitors in solid tumors (<https://clinicaltrials.gov/>) [44]. Moreover, although they have not been tested in ACC patients yet, CDK4/6 inhibitors have been shown to reduce cell viability in ACC cell lines [45 46].

Another promising drug target is *NOTCH1* gene, which was gained in >20% of cases in present series and in >40% in a previous study [47]. Notch pathway might represent an interesting target as it was reported to be activated in ACC [48] and can be actioned by different gamma secretase inhibitors (GSI) or monoclonal antibodies [49]. For instance, the GSI PF-03084014 has already been tested in Phase I study in patients with advanced solid tumors [50]. Presence of copy number gains at MDM2 (7% of cases) might also be considered encouraging targets as MDM2 inhibitors such as DS-3032b or RG7112 have been reported to reduce cell proliferation in MDM-amplified liposarcoma [51]. An interesting therapeutic option is also represented by targeting the BRCA-related DNA repair system (altered in >7% of cases) by PARP inhibitors (i.e. olaparib, nirapanib and rucaparib) [52] that are approved for treatment of BRCA-mutant ovarian. Moreover, mutations in targetable genes coding for receptor tyrosine kinases (*EGFR*, *KIT*, *RET*), members of mTOR pathway (*TSC1/2*) and *BRAF* were detected in rare cases. Finally, in 4.5% of cases, we observed mutations or CN losses in members of the MMR system (*MSH2*, *MSH6*, *MLH1*), which have been reported as predictive biomarkers for antitumor effects of checkpoint PD1/PDL1 inhibitors (i.e. pembrolizumab or novolumab [53 54]). Our findings on actionable targets open up new therapeutic avenue for subsets of ACC patients.

462 In conclusion, our study demonstrates that molecular classification based on targeted genetic analysis
463 is able to improve the prognostication of ACC patients when combined with clinical/histopathological
464 parameters. This approach paves the way to a personalized management of ACC, allowing better
465 decisions about need for adjuvant therapies and/or frequency of periodical post-operative monitoring.
466 Additionally, our targeted panel can at the same time identify druggable targets. In some cases, these
467 results may be used to select patients for clinical trials or off-label use of specific anti-cancer drugs.
468 The fact that all this is possible in readily available FFPE material is a major step towards precision
469 medicine in this rare disease.

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Legend to figures

Figure 1. Recurrent genetic alterations observed by targeted next generation sequencing and Sanger sequencing (*ZNRF3*) in adrenocortical carcinoma. A) Somatic protein-altering mutations detected in the present series of FFPE tumors (n=107) and in the series of snap-frozen tumors available in the literature (n=182); B) Copy number (CN) alterations (gains and losses) detected in the present series of FFPE tumors (n=107). Heterozygosis=duplicated with a fold change between 1.25 and 1.75, homozygosis=duplicated with a fold change > 1.75. C) Altered signaling pathways identified by curated analysis. Alterations are defined by somatic mutations and copy number gains or losses.

Figure 2. Example of CDK4 copy number (CN) gains. A) CN analysis by targeted next generation sequencing in one FFPE tumor slide with homozygous CDK4 amplification (view of all genes covered in the panel); B) zoom view of the CDK4 locus at chr. 12); and C) validation by FISH analysis in the same FFPE tumor slide (Dual Color Probe: CDK4 in green and CEN12 in red). D) CN analysis by targeted next generation sequencing in one FFPE tumor slide with homozygous CDK4 amplification (view of all genes covered in the panel); E) zoom view of the CDK4 locus at chr. 12); and F) validation by FISH analysis, respectively. G) CN analysis by targeted next generation sequencing in one FFPE tumor slide without CDK4 amplification (view of all genes covered in the panel); H) zoom view of the CDK4 locus at chr. 12); and I) validation by FISH analysis, respectively.

Figure 3. Prognostication of clinical outcome (progression-free survival) in 107 patients with adrenocortical carcinoma according to different parameters. A) Initial ENSAT tumor stage (1 to 4); B) Modified GRAS score including ENSAT tumor stage (1 or 2=0, 3=1, 4=2), Ki67 index (0-9=0, 10-19=1, ≥ 20 =2), R status (R0=0, RX=1, R1=2, R2=3), age (<50 years=0, ≥ 50 years=1) and symptoms (no=0, yes=1) (overall points 0-9); C) Molecular score including number of somatic mutations (0-1=0, >1=1), alterations in the Wnt/ β -catenin and p53/Rb pathways (none=0, only Wnt/ β -catenin=1, Wnt/ β -catenin+ p53/Rb=2) and promoter regions methylation pattern ($\leq 25\%$ =0, >25%=1) (overall points 0 to 4); D) COMBI score including mGRAS and molecular score (overall points 0 to 13).

Figure 4. Heatmap representing the clinical outcome of the 107 patients with adrenocortical carcinoma in relationship with the evaluated clinical/histopathological score (mGRAS), the most relevant and “easy-to-apply” molecular parameters and the calculated COMBI score. Patients are subdivided into four different “prognosis” subgroups according to PFS: favorable prognosis by PFS >24 months (n=29), good prognosis by PFS 12-24 months (n=13), intermediate prognosis by PFS 6-11 months (n=20) and bad prognosis by PFS <6 months (n=40). The remaining 5 patients who were still alive at the last follow up were considered as “not applicable” in terms of prognosis classification because of an insufficient time-span of follow up.

Legend to Tables

Table 1. Baseline clinical/histopathological characteristics of 107 patients with adrenocortical carcinoma included in the study.

Table 2. Potential drug targets identified by next-generation sequencing in 107 patients with adrenocortical carcinomas.

Table 1. Baseline clinical/histopathological characteristics of 107 patients with adrenocortical carcinoma included in the study.

| Parameter | |
|--|------------|
| N | 107 |
| Sex (M/F) | 46/61 |
| Baseline (at time of diagnosis) | |
| Age – yrs (median, range) | 49 (18-87) |
| - <50 years – n (%) | 57 (53.3) |
| - ≥50 years – n (%) | 50 (46.7) |
| Clinical presentation (available data) | 107 |
| - Incidentally – n (%) | 31 (29.0) |
| - Tumor-related – n (%) | 45 (42.0) |
| - Hormone-related – n (%) | 31 (29.0) |
| Hormone secretion (available data) | 81 |
| - Glucocorticoids alone – n (%) | 24 (29.6) |
| - Mixed secretion – n (%) | 25 (30.8) |
| - Endocrine inactive – n (%) | 25 (30.8) |
| - Others (androgens, mineralcorticoids, estrogens) – n (%) | 7 (8.6) |
| - Unknown – n | 26 |
| Initial ENSAT tumor stage (available data) | 107 |
| 1-2 – n (%) | 56 (52.3) |
| 3 – n (%) | 28 (26.2) |
| 4 (metastatic) – n (%) | 23 (21.5) |
| Resection status (available data) | 104 |
| R0 – n (%) | 74 (71.1) |
| RX – n (%) | 16 (15.4) |
| R1 – n (%) | 5 (4.8) |
| R2 – n (%) | 9 (8.6) |
| Unknown - n | 3 |
| Ki67 proliferation index (median, range) | 12 (1-90) |
| - 0-9 – n (%) | 31 (29.0) |
| - 10-19 - n (%) | 33 (30.8) |
| - ≥20 – n (%) | 43 (40.2) |
| Type of tumor | |
| Primary – n (%) | 89 (83.2) |
| Local recurrence – n (%) | 9 (8.4) |
| Metastasis – n (%) | 9 (8.4) |
| During follow up | |
| Duration of follow up – months (median, range) | 31 (3-274) |
| Deaths – n (%) | 54 (50.5) |
| Local therapeutic approaches | |
| Additional surgeries – n (%) | 26 (24.3) |
| Radiotherapy (tumor bed or metastasis) – n (%) | 19 (17.8) |
| Iodometomidate – n (%) | 7 (6.5) |
| Mitotane | 73 (68.2) |
| Adjuvant setting – n (%) | 39 (36.4) |
| Palliative setting – n (%) | 34 (31.8) |
| Cytotoxic chemotherapies | |
| None – n (%) | 45 (42.1) |
| Platinum-compounds – n (%) | 52 (48.6) |
| Streptozotocin – n (%) | 44 (41.1) |
| Gemcitabin plus Capecitabin – n (%) | 36 (33.6) |

Abbreviations: F=female; M=male; n=number of patients; n.a= not available; ns=not significant; R0=complete resection; R1=microscopic incomplete resection; R2=macroscopic incomplete resection; RX=uncertain resection; yrs=years.

Table 2. Potential drug targets identified by next-generation sequencing in 107 patients with adrenocortical carcinomas.

| Gene symbol | Description | Type of observed alteration | % samples | Potential targeted therapy | Level of evidence ^A |
|------------------|------------------------------|------------------------------|-----------|---|--|
| DNA level | | | | | |
| <i>CDK4</i> | Cyclin dependent kinase | CN gains | 43 | CDK4/6 inhibitor (palbociclib/abemaciclib/ribociclib) | 2A (liposarcoma) |
| <i>NOTCH1</i> | NOTCH signaling | Missense mutation | 1.8 | Gamma secretase inhibitor (PF-03084014) | 4 (all tumours) |
| | | CN gains | 20.5 | | |
| <i>NF1</i> | RAS/MAPK regulation | Del/Dup or missense mutation | 11.2 | MEK inhibitor (trametinib/cobimetinib) | 4 (glioblastoma/melanoma) ^B |
| <i>MDM2</i> | P53 pathway | CN gain | 7.0 | MDM2 inhibitors (DS-3032b, RG7112) | 3A (liposarcoma) |
| <i>EGFR</i> | Receptor tyrosin kinase | Missense mutation | 2.8 | TKI (afatinib/erlotinib/gefitinib) | 1 (NSCLC) ^B |
| <i>BRCA1</i> | DNA repair system | Del or missense mutation | 2.8 | PARP inhibitor (rucaparib/olaparib/nirapanib) (synthetic lethality) | 1-2A (ovary ca) ^B |
| <i>BRCA2</i> | | Missense mutation | 2.8 | | |
| <i>ATM</i> | DNA repair system | Missense mutation or delins | 1.8 | PARP inhibitor (olaparib) (synthetic lethality) | 4 (prostate ca) ^B |
| <i>BRAF</i> | Ser/thr kinase | Missense mutation | 1.8 | BRAF inhibitor (vemurafenib/dabrafenib) MEK inhibitor (trametinib/cobimetinib) | 1 (cutaneous melanoma) 2A (NCSLC) 4 (thyroid ca) |
| <i>PTCH1</i> | Sonic hedgehog receptor | Missense mutation | 1.8 | Hedgehog inhibitor (sonidegib) | 3A (skin ca) ^B |
| <i>TSC1</i> | mTOR pathway | Del (frameshift) | 1.8 | mTOR inhibitor (everolimus) | 2A (CNS + renal ca) ^B |
| <i>TSC2</i> | | Missense mutation | 0.9 | | |
| <i>KIT</i> | Receptor tyrosin kinase | Missense mutation | 0.9 | TKI (imatinib/sunitinib) | 1 (GIST) ^B 2A (melanoma) |
| <i>RET</i> | Receptor tyrosin kinase | Missense mutation | 0.9 | TKI (cabozantinib) | 2A (NSCLC) ^B |
| <i>ESR1</i> | Estrogen receptor | Missense mutation | 0.9 | AZD9496 (fulvestrant) | 3A (breast ca) ^B |
| <i>EZH2</i> | Histone N-methyl-transferase | Nonsense mutation | 0.9 | GSK126 (tazemetostat) | 4 (B-cell lymphoma) ^B |

Abbreviations: CN=copy number; NCSLC=non-small cell lung cancer; TKI=tyrosine kinase inhibitor.

^AEvidence by OncoKB website (<http://oncokb.org/#/>, (21)): Level 1=FDA-approved biomarker; Level 2A= standard care biomarker in this indication; Level 2B: Standard care biomarker in another indication, Level 3A= predictive biomarker according to clinical evidence in this indication; Level 3B= predictive biomarker according to clinical evidence in another indication Level 4= predictive biomarker according to biological evidence. ^Bnot the same molecular alteration.

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Frequency somatic mutations (%)

■ Somatic mutations (current series, n=107)
■ Somatic mutations (literature, n=182)

| Gene | Current Series (%) | Literature (%) |
|--------|--------------------|----------------|
| AR | 1.0 | 1.0 |
| ATM | 1.0 | 1.0 |
| BRCA1 | 1.0 | 1.0 |
| CDK12 | 1.0 | 1.0 |
| CSF1B | 1.0 | 1.0 |
| EP300 | 1.0 | 1.0 |
| ESR1 | 1.0 | 1.0 |
| FANCE | 1.0 | 1.0 |
| FBXO11 | 1.0 | 1.0 |
| GNA1 | 1.0 | 1.0 |
| H3F3A | 1.0 | 1.0 |
| JAK1 | 1.0 | 1.0 |
| MAP3 | 1.0 | 1.0 |
| MED12 | 1.0 | 1.0 |
| MYC | 1.0 | 1.0 |
| NOTC | 1.0 | 1.0 |
| REL | 1.0 | 1.0 |
| PTPN11 | 1.0 | 1.0 |
| PTEN | 1.0 | 1.0 |
| SCTD4 | 1.0 | 1.0 |
| SMA | 1.0 | 1.0 |
| TERT | 1.0 | 1.0 |
| TSC2 | 1.0 | 1.0 |
| ABL1 | 1.0 | 1.0 |
| ATM | 1.0 | 1.0 |
| BRCA1 | 1.0 | 1.0 |
| BRCA2 | 1.0 | 1.0 |
| BRF1 | 1.0 | 1.0 |
| CDH1 | 1.0 | 1.0 |
| DNM1 | 1.0 | 1.0 |
| FBXW7 | 1.0 | 1.0 |
| GNA12 | 1.0 | 1.0 |
| JAK2 | 1.0 | 1.0 |
| KMT2D | 1.0 | 1.0 |
| MLL1 | 1.0 | 1.0 |
| INSH2 | 1.0 | 1.0 |
| PRMT1 | 1.0 | 1.0 |
| PRK2 | 1.0 | 1.0 |
| PTCH1 | 1.0 | 1.0 |
| SEL1D2 | 1.0 | 1.0 |
| U2AF | 1.0 | 1.0 |
| BRCA1 | 1.0 | 1.0 |
| BEGF | 1.0 | 1.0 |
| KDM6A | 1.0 | 1.0 |
| KOR | 1.0 | 1.0 |
| RBI | 1.0 | 1.0 |
| DAXX | 1.0 | 1.0 |
| ATRX | 1.0 | 1.0 |
| GNAS | 1.0 | 1.0 |
| NR1H3 | 1.0 | 1.0 |
| APC | 1.0 | 1.0 |
| CTNNB1 | 1.0 | 1.0 |
| TP53 | 1.0 | 1.0 |

Frequency copy number alterations (%)

Legend:

- CN loss heterozygosity %
- CN gain heterozygosity %
- CN gain homozygosity %

| Gene | CN loss heterozygosity % | CN gain heterozygosity % | CN gain homozygosity % | Total Frequency (%) |
|----------|--------------------------|--------------------------|------------------------|---------------------|
| ABL1 | 0 | 2 | 0 | 2 |
| CDC73 | 2 | 0 | 0 | 2 |
| CSF1R | 1 | 1 | 0 | 2 |
| ECT2L | 2 | 0 | 0 | 2 |
| EPCAM | 2 | 0 | 0 | 2 |
| EZH2 | 1 | 1 | 0 | 2 |
| MAP3K1 | 1 | 1 | 0 | 2 |
| MLH1 | 2 | 0 | 0 | 2 |
| NPM1 | 1 | 1 | 0 | 2 |
| PHF6 | 2 | 0 | 0 | 2 |
| SPOP | 2 | 0 | 0 | 2 |
| FGFR2 | 3 | 0 | 0 | 3 |
| MSH2 | 3 | 0 | 0 | 3 |
| SLC7A8 | 2 | 1 | 0 | 3 |
| VHL | 3 | 0 | 0 | 3 |
| GNAQ | 3 | 0 | 0 | 3 |
| GNAS | 3 | 1 | 0 | 4 |
| PIK3R1 | 2 | 1 | 1 | 4 |
| SDHB1 | 3 | 0 | 0 | 3 |
| BRIP1 | 4 | 0 | 0 | 4 |
| CIC | 0 | 4 | 0 | 4 |
| IL7R | 1 | 3 | 1 | 5 |
| RB1 | 5 | 0 | 0 | 5 |
| SMAD4 | 5 | 0 | 0 | 5 |
| MDM2 | 1 | 6 | 1 | 8 |
| MAP2K4 | 10 | 0 | 0 | 10 |
| FGFR3 | 4 | 7 | 1 | 12 |
| TERT | 0 | 7 | 6 | 13 |
| CHEK2 | 13 | 0 | 0 | 13 |
| GNA11 | 0 | 4 | 13 | 17 |
| FLCN | 19 | 0 | 0 | 19 |
| NOTCH1 | 0 | 16 | 3 | 19 |
| SMARCB1 | 22 | 0 | 0 | 22 |
| TNFRSF14 | 30 | 0 | 0 | 30 |
| STK11 | 4 | 26 | 1 | 31 |
| CDK4 | 0 | 36 | 7 | 43 |

p53/Rb1 signaling
59.8% of cases altered

CDKN2A
0%
Deleted NA, mutated 0%

CDK4
44.8%
Amplified 43%, mutated 1.8%

MDM2
7.6%
Amplified

RB1
9.9%
Deleted 5.2%, mutated 4.7%

TP53
22.4%
Mutated

Cell cycle progression

DNA mismatch repair
4.5% of cases altered

MLH1
1.8%
Mutated

MSH2
1.8%
Mutated

MSH6
0.9%
Mutated

DNA damage response/
genomic stability

Chromatin remodelling
29.9% of cases altered

ATRX
6.5%
Mutated

DAXX
4.7%
Mutated

KDM6A
2.8%
Mutated

SETD2
1.8%
Mutated

MEN1
7.4%
Mutated

DNMT3A
1.8%
Mutated

TERT
12.4%
Amplified

KMT2D
1.8%
Mutated

Wnt/beta catenin
33.6% of cases altered

ZNRF3
8.4%
Deleted NA, mutated 8.4%

APC
8.4%
Mutated

CTNNB1
16.8%
Mutated

Cell cycle progression

NOTCH-Signalling
27.1% of cases altered

FBXW7
1.9%
Mutated

NOTCH1
25.2%
Amplified 20.5%, mutated 4.7%

Cell-cell communication

BRCA-DNA repair
7.4% of cases altered

ATM
1.8%
Mutated

BRCA1
2.8%
Mutated

BRCA2
2.8%
Mutated

DNA damage response/
genomic stability

Percentage of Cases (%)

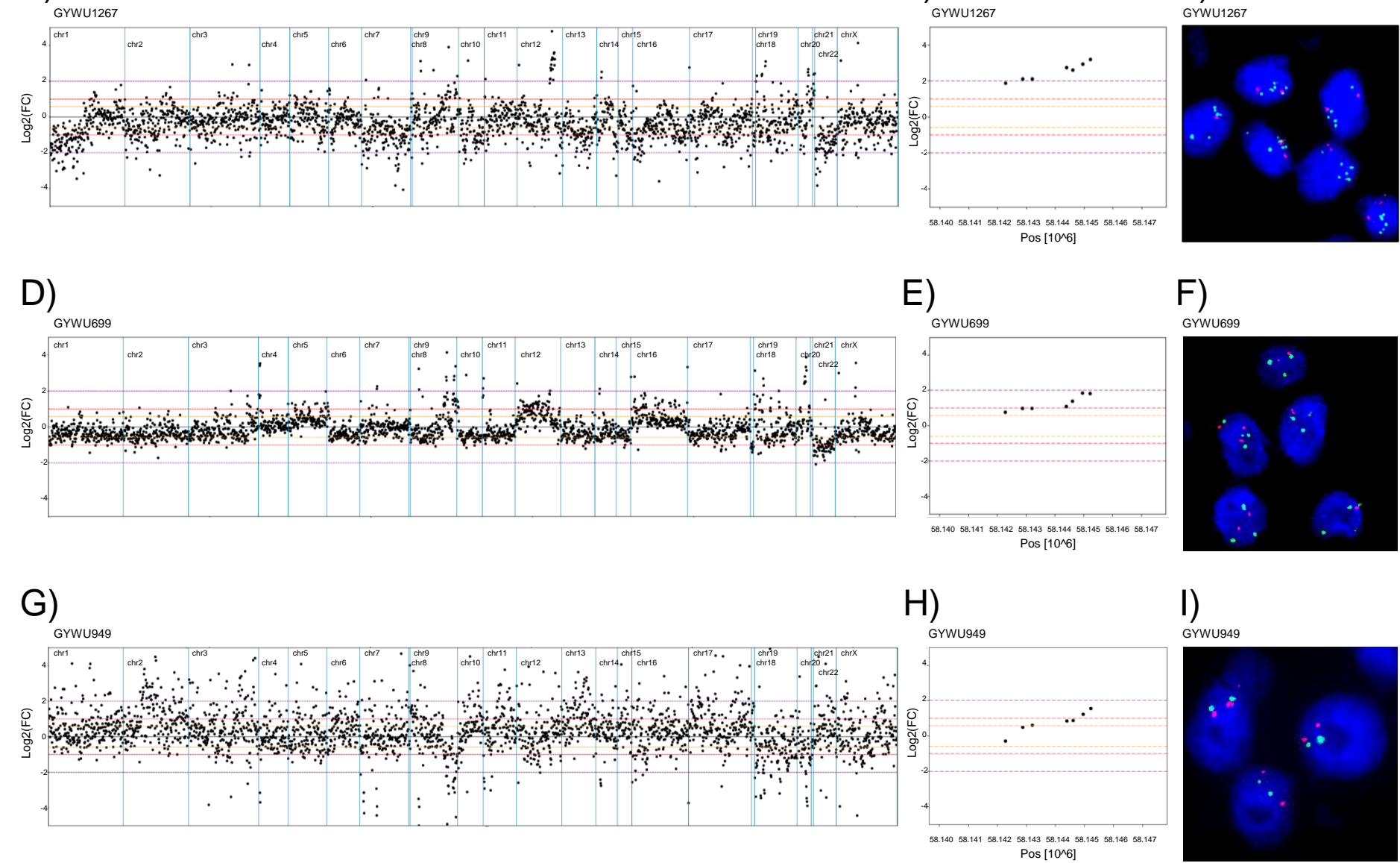
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Inactivated Activated

Variable

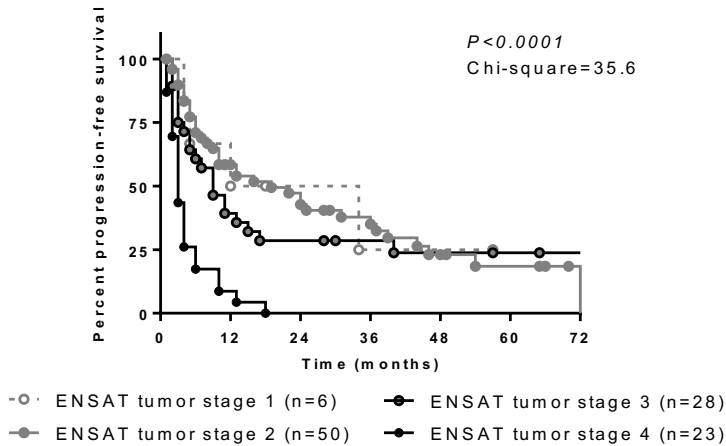
Inhibition Activation

Figure 2

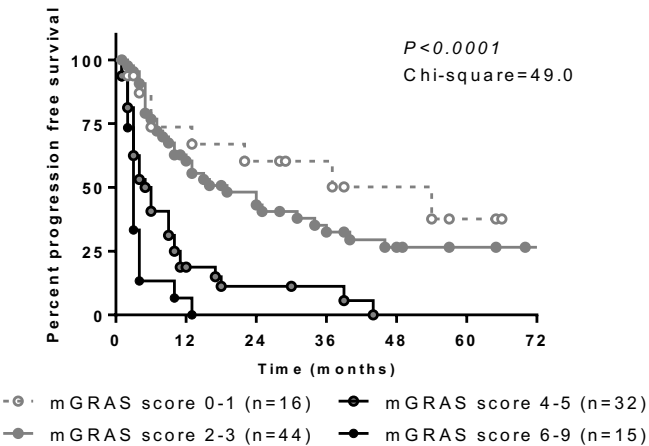
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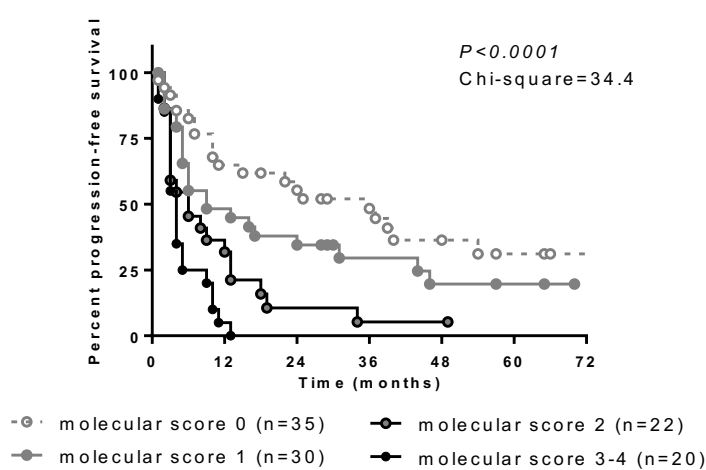
A)



B)



C)



D)

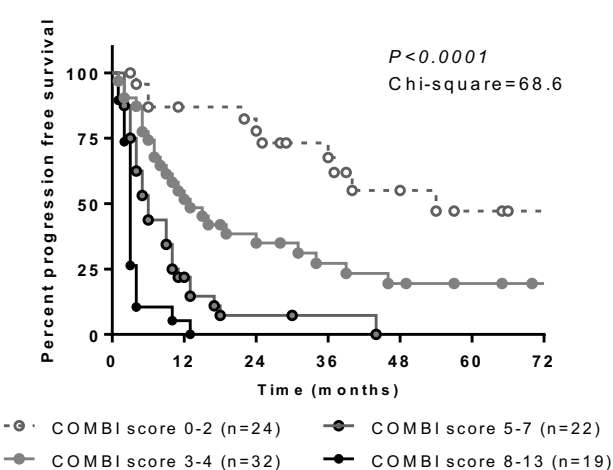
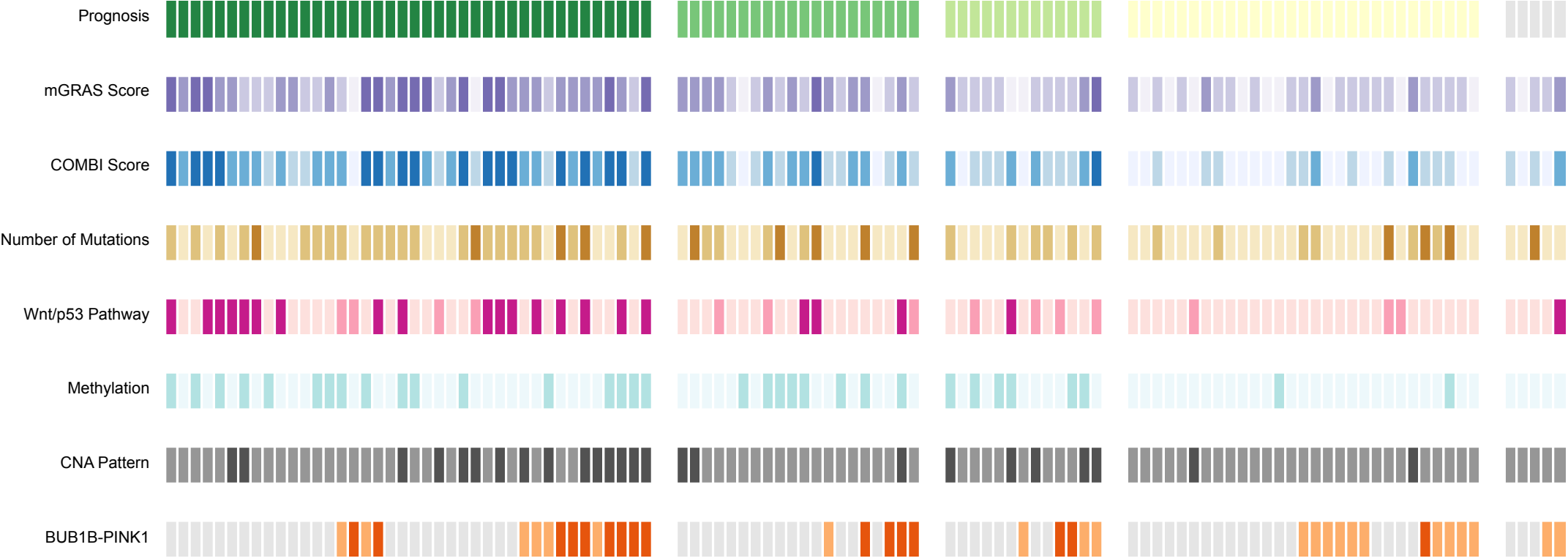


Figure 4



Legend:

| | | | | | |
|----------------------|---------|-------------------|---------------|------|----------------|
| Prognosis: | bad | intermediate | good | best | not applicable |
| mGRAS Score: | 0-1 | 2-3 | 4-5 | 6-8 | |
| COMBI Score: | 0-2 | 3-4 | 5-7 | >7 | |
| Number of Mutations: | 0-1 | 2-4 | >4 | | |
| Wnt/p53 Pathway: | none | Wnt only | Wnt and p53 | | |
| Methylation: | <= 25 % | > 25 % | | | |
| CNA Pattern: | noisy | chromosomal/quiet | | | |
| BUB1B-PINK1: | <= 6.3 | >6.3 | not available | | |



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Supplemental Material

20180716_Supplemental Figures.docx